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A New Oxygen Sensitivity and Its Potential Application in Photosynthetic H_2 Production

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Abstract

We have discovered a new competitive pathway for O_2 sensitivity in algal H_2 production that is distinct from the O_2 sensitivity of hydrogenase *per se*. This O_2 sensitivity is apparently linked to the photosynthetic H_2 production pathway that is coupled to proton translocation across the thylakoid membrane. Addition of the proton uncoupler carbonyl cyanide-p-trifluoromethoxy-phenylhydrazone eliminates this mode of O_2 inhibition on H_2 photoevolution. This newly discovered inhibition is most likely owing to background O_2 that apparently serves as a terminal electron acceptor in competition with the H_2 production pathway for photosynthetically generated electrons from water splitting. This O_2-sensitive H_2 production electron transport pathway was inhibited by 3[3,4-dichlorophenyl]1,1-dimethylurea. Our experiments demonstrated that this new pathway is more sensitive to O_2 than the traditionally known O_2 sensitivity of hydrogenase. This discovery provides new insight into the mechanism of O_2 inactivation of hydrogenase and may contribute to the development of a more-efficient and robust system for photosynthetic H_2 production.

Index Entries: Oxygen sensitivity; H_2 production; photosynthetic H_2 production; H_2 production pathways; hydrogenase.

Introduction

Algal photosynthetic hydrogen (H_2) production by light-activated water splitting is a potentially clean energy resource. However, compared to our knowledge of the pathway of atmospheric CO_2 reduction, and in spite of the potential importance of the hydrogen-producing reaction, relatively little is known concerning the mechanistic pathway of electron flow in hydrogen-producing algae. In green algae, such as *Chlamydomonas reinhardtii*, photoevolution of H_2 and O_2 occurs in the same cell, where the photosynthetically produced O_2 can inhibit the production of H_2 *(1)*. There-

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fore, the application of green algae for H_2 production must address the problem of O_2 sensitivity. Historically, this O_2-sensitive phenomenon was generally interpreted as the direct O_2 inhibition of hydrogenase activity *(2)*. We report here that the classic interpretation of O_2 sensitivity should be revised. In recent experiments characterizing O_2 tolerance in H_2-producing wild-type *C. reinhardtii*, we observed a new O_2 sensitivity that is clearly distinct from that of classic O_2 inhibition of hydrogenase. The O_2 sensitivity indicates that there is a competitive electron transport pathway that can redirect electrons from the hydrogenase-catalyzed H_2 production pathway to O_2. That is to say, suppression of H_2 evolution in the presence of low-level background concentrations of O_2 is owing to the drain of reducing equivalents away from the hydrogenase pathway and toward the reduction of O_2. Our experiments demonstrated that the competitive pathway mechanism is more sensitive to O_2 than the classic O_2 sensitivity of hydrogenase and can be suppressed by proton uncoupler carbonyl cyanide-p-trifluoro-methoxyphenylhydrazone (FCCP).

Materials and Methods

First evidence of the new O_2 sensitivity was obtained from H_2 production assays in *C. reinhardtii* wild-type strain 137c. The assays were conducted using a laboratory-built dual-reactor flow detection system *(3)*. For each assay, 35 mL of algal sample (3 µg Chl/mL) was placed and sealed in each of the two water-jacketed reactors and held at 20°C with a temperature-controlled water bath. The algal sample was purged with a helium flow (50 mL/min) through the liquid reaction medium. The helium flow served two purposes: (1) to remove O_2 from the algal sample to establish and maintain anaerobic conditions that are necessary for induction of the algal hydrogenase activity and production of H_2, and (2) to carry the photoproduced H_2 gas product to the hydrogen sensors. After induction of hydrogenase and establishment of steady-state photoevolution of H_2 under the helium atmosphere (which requires about 8 h), a primary standard of 1000 ppm of O_2 in helium replaced the pure helium at the same flow rate (50 mL/min) to characterize the oxygen sensitivity of photoevolution of H_2. Actinic illumination of 100 µE/(m^2·s) for the H_2 photoevolution assay was provided by an electronically controlled light emitting diode (LED) light source at a wavelength of 670 nm. As illustrated in Fig. 1, introduction of 0.1000% (1000 ppm) of O_2 dramatically reduced the rate of algal H_2 photoevolution. The steady-state H_2 production rate in the presence of 0.1000% O_2 was 0.33 µmol of H_2·mg of Chl^{-1}·h^{-1}, which is only about 2.8% of the full steady-state rate (12 µmol H_2·mg of Chl^{-1}·h^{-1}) before the introduction of the 0.1000% O_2. In the past, this type of H_2 production decay was commonly interpreted as the inhibition of hydrogenase activity by O_2. Our results prove that this classic interpretation of oxygen sensitivity on algal H_2 production is not consistent with the data. According to the classic interpretation, the decrease in H_2 production after the introduction of 0.1000%

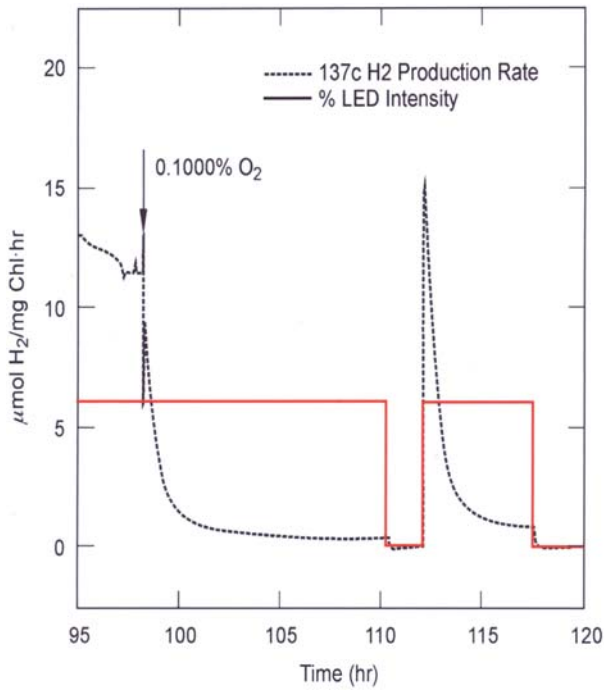

Fig. 1. Observation of a new O_2 sensitivity to algal H_2 production in *C. reinhardtii*.

O_2 is owing to O_2 inhibition of hydrogenase *per se*: that is, loss of hydrogenase activity is the limiting factor for the rate of H_2 photoevolution. If this interpretation were correct, after a brief dark period in the presence of 0.1000% O_2, one would expect the rate of H_2 photoevolution to be no higher than the inhibited rate (0.33 μmol of H_2·mg of Chl^{-1}·h^{-1}) preceding the dark interval. However, the data are quite different from the classic expectation. As shown in Fig. 1, there was a surge of H_2 photoevolution after a 2-h dark period *in the continuous presence of 0.1000% O_2*. The peak rate of H_2 photoevolution was about 15 μmol of H_2·mg of Chl^{-1}·h^{-1}, which is about 45 times higher than the classically predicted rate (0.33 μmol of H_2·mg of Chl^{-1}·h^{-1}) and well outside the experimental error of the measurement. This assay has now been repeated six times, and all results were consistent with the observation presented in Fig. 1.

This observation clearly indicated that hydrogenase activity was not the limiting factor for H_2 photoevolution at this level of O_2. There must be an alternative electron transport pathway that takes the photogenerated electrons away from ferredoxin (Fd) to O_2. The observed reduction of H_2 production after the introduction of 0.1000% O_2 can be explained by such a pathway that competes for electrons with the Fd/hydrogenase-catalyzed H_2 production pathway. This is a significant discovery since it fundamentally redefines the meaning of "oxygen tolerance" in algal H_2 production.

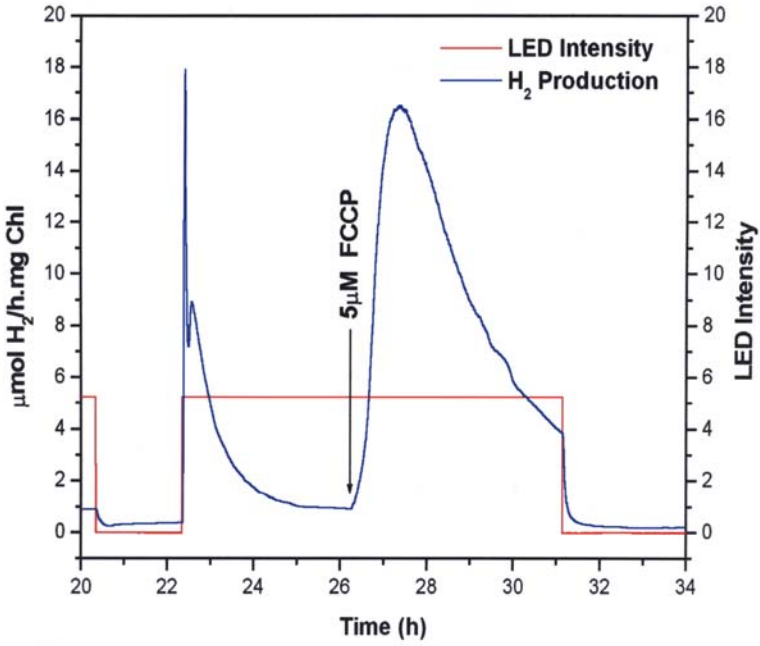

Fig. 2. Stimulation of photosynthetic H_2 production of *C. reinhardtii* 137c following addition of proton uncoupler FCCP in background atmosphere of 1000 ppm of O_2. Addition of 5 μ*M* FCCP produced a dramatic increase in H_2 production followed by a slow decay. The slow decay was owing to a side effect of FCCP known as ADRY, in which FCCP gradually inhibits PSII activity. This experimental result indicates that use of a polypeptide proton channel that does not have the ADRY effect could enhance H_2 production by eliminating the problems of both the proton gradient accumulation and the newly discovered alternative O_2 sensivity.

Studies with the chemical inhibitor 3[3,4-dichlorophenyl]1,1-dimethylurea (DCMU) and the proton uncoupler FCCP yielded additional evidence for the new O_2 sensitivity. FCCP is a proton uncoupler that can dissipate the proton gradient across the thylakoid membrane in algal cells. As illustrated in Fig. 2, in the presence of 1000 ppm of O_2 after the induction of the hydrogenase enzyme, the steady-state photoevolution of H_2 around the time of 20 h was slightly less than 1 μmol of H_2·mg of Chl^{-1}·h^{-1} (cf. Fig. 1 at approx 110 h). After a brief dark period (from 20:20 to 22:20), a burst of H_2 photoevolution appeared, followed by an oscillation in the decay curve. Since both the actinic intensity and the background O_2 concentration (1000 ppm) remained the same, this H_2 production oscillation also indicated that the decay in the rate of H_2 photo-evolution resulted not from O_2 inhibition of the hydrogenase enzyme *per se*, but from a competitive kinetic effect of O_2 on electron transport that is related to the H_2 production process. The addition of 5 μ*M* FCCP produced a dramatic removal of O_2 inhibition on H_2 photoevolution. The rate of H_2 production rose to about 16 μmol of H_2·mg of Chl^{-1}·h^{-1}. This FCCP-stimulated H_2 production is clearly photo-dependent.

As soon as the actinic light was turned off, H_2 production stopped. The data (Fig. 2) also demonstrated that FCCP-enhanced photoevolution of H_2 can last for more than 4 h, although at a decreasing rate. The decrease in rate of H_2 photoproduction is owing to a secondary effect of FCCP known in the photosynthesis research literature as the acceleration of the deactivation reactions of the water-splitting system Y (ADRY) effect, in which FCCP gradually inhibits photosystem II (PSII) activity by deactivating the photosynthetic water-splitting complex in the S_2 and S_3 states *(4)*. However, FCCP does not have any known effect on hydrogenase *per se*. Therefore, the observed stimulation of H_2 photoevolution by FCCP in the presence of 1000 ppm of O_2 clearly demonstrated that the newly discovered O_2-sensitive electron transport pathway requires the presence of a proton gradient (or ATP) to operate.

DCMU is a chemical inhibitor that binds at the Q_B site of PSII and blocks transport of electrons acquired from PSII water splitting to photosystem I. The experimental data (not shown) demonstrated that the addition of DCMU inhibited the burst of H_2 photoevolution after a dark period in the presence of 1000 ppm of background O_2. This result indicated that > 90% of the electrons that are used in the photoproduction of H_2 are derived from PSII water splitting. Therefore, water is the main source of electrons for the H_2 burst after the dark period in the presence of 1000 ppm of O_2. Organic reserves such as starch are thus not the main source of electrons in this mode of H_2 production.

The new O_2 sensitivity was further characterized using a series of O_2 concentrations: 10, 100, 300, 1000, 5000, and 10,000 ppm of O_2 in helium. The experimental results showed that the introduction of 100 ppm of O_2 had no significant effect on the steady-state rate of H_2 photoevolution in *C. reinhardtii* wild-type strain 137c. However, the addition of 300 ppm of O_2 began to show an effect. Figure 3 plots the percentage of steady-state H_2 production rate vs background O_2 concentrations in the wild-type 137c. The O_2 concentration that gave 50% inhibition of H_2 photoevolution was about 500 ppm. When the O_2 concentration was raised to 5000 ppm, the inhibition on H_2 production was dramatic and the rate of H_2 photo-evolution decreased to nearly zero (Figs. 3 and 4). However, the hydrogenase in the algal cells was still active even after the continued presence of 5000 ppm of O_2 for more than 10 h. When the actinic was turned on again after hour 198, a small peak of H_2 photoproduction was observed. As illustrated in the expanded scale (Fig. 4B), this H_2 photoproduction peak was clearly above the background noise and/or dark H_2 signal, indicating the presence of active hydrogenase in the algal cells. Therefore, hydrogenase in the wild-type cells can tolerate up to 5000 ppm of O_2. The newly discovered O_2 sensitivity is about 10 times more sensitive to O_2 than that of the hydrogenase.

Results and Discussion

The new O_2 sensitivity is apparently linked to the photosynthetic H_2 production pathway that is coupled with proton translocation across the

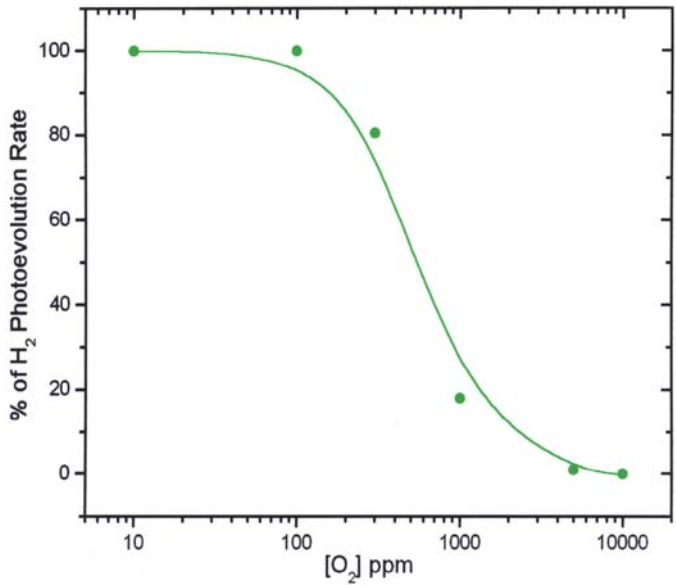

Fig. 3. Effect of background O_2 concentrations on steady-state H_2 photoevolution.

thylakoid membrane. The addition of the proton uncoupler FCCP can eliminate this mode of O_2 inhibition on H_2 photoevolution. This O_2 inhibition on H_2 production is most likely owing to the competitive uptake of reducing equivalents by background O_2, with the H_2 production pathway for photosynthetically generated electrons from water splitting. The O_2-sensitive H_2 production pathway can be inhibited by DCMU. Our experiments demonstrated that the competitive pathway is more sensitive to O_2 than the classic O_2 sensitivity of hydrogenase. These findings redefine the meaning of "oxygen tolerance" in algal H_2 production. As discussed, this O_2 sensitivity apparently represents a new pathway in the photosynthetic H_2 production that is coupled with proton translocation across the thylakoid membrane. As illustrated in Fig. 5A, the site for the reduction of O_2 could be at the RuBisco enzyme, which can serve as an RuDP (also known as RuBP) carboxylase and/or an RuDP oxygenase in the Calvin cycle. Under conditions for H_2 photoevolution in which CO_2 is not present and ATP is abundant owing to associated photophosphorylation, the Calvin cycle enzymes are fully activated and RuBisco could act as a strong oxygenase. This hypothesis can explain how FCCP mitigates O_2 inhibition of H_2 photoevolution, since operation of the Calvin cycle requires formation of ATP using the proton gradient across the thylakoid membrane. Another possible site for O_2 interaction could be at Fd, which, according to the classic Mehler reaction, can serve as an electron donor to O_2. Additional experimental studies with different chemical inhibitors and genetic mutants are under way to elucidate this O_2-sensitive H_2 production electron transport pathway.

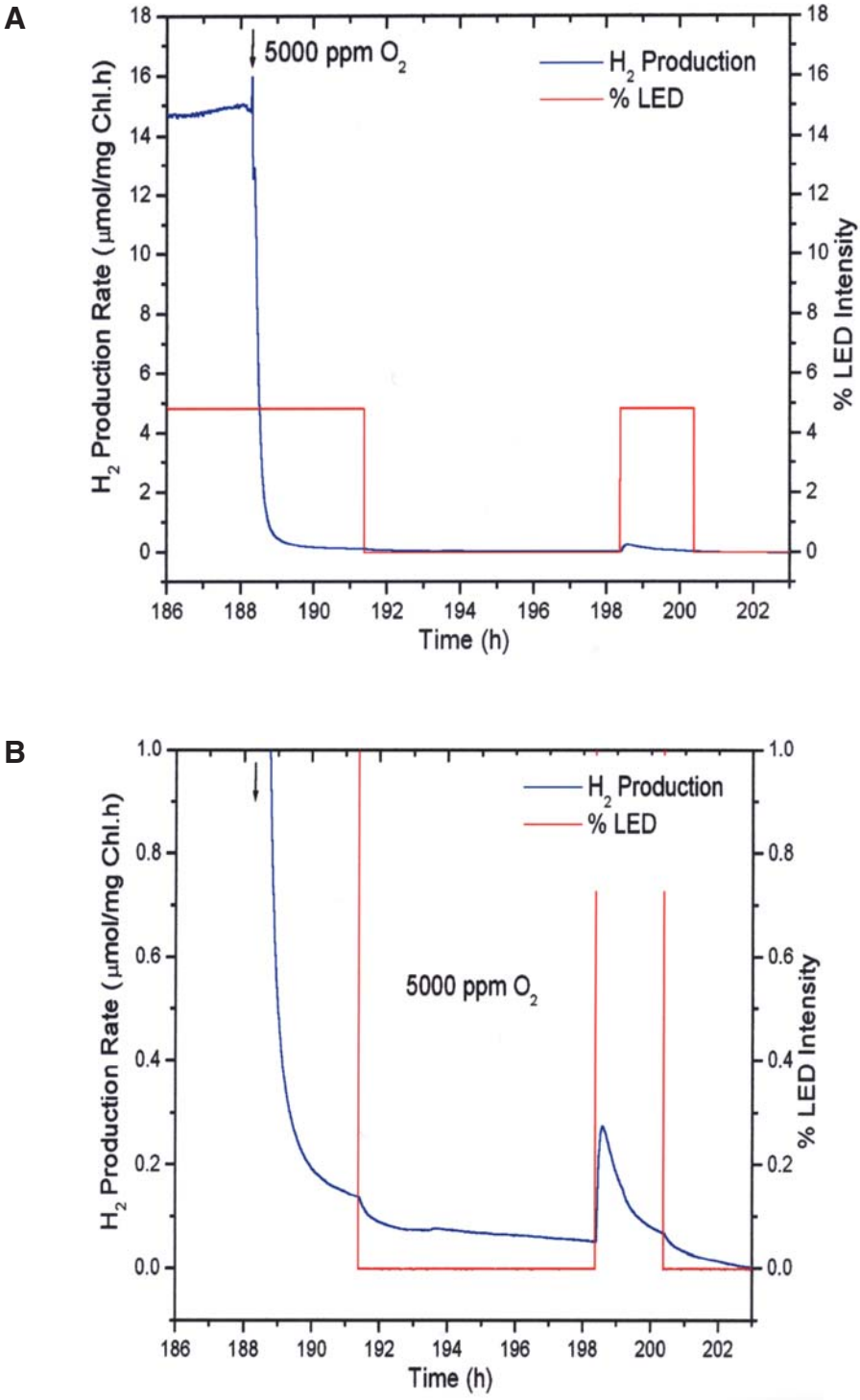

Fig. 4 **(A)** The introduction of 5000 ppm of O_2 dramatically inhibits H_2 production, but the hydrogenase remains active. **(B)** Expanded vertical scale of (A), showing clear peak of H_2 photoevolution after 10 h of continued presence of 5000 ppm of O_2.

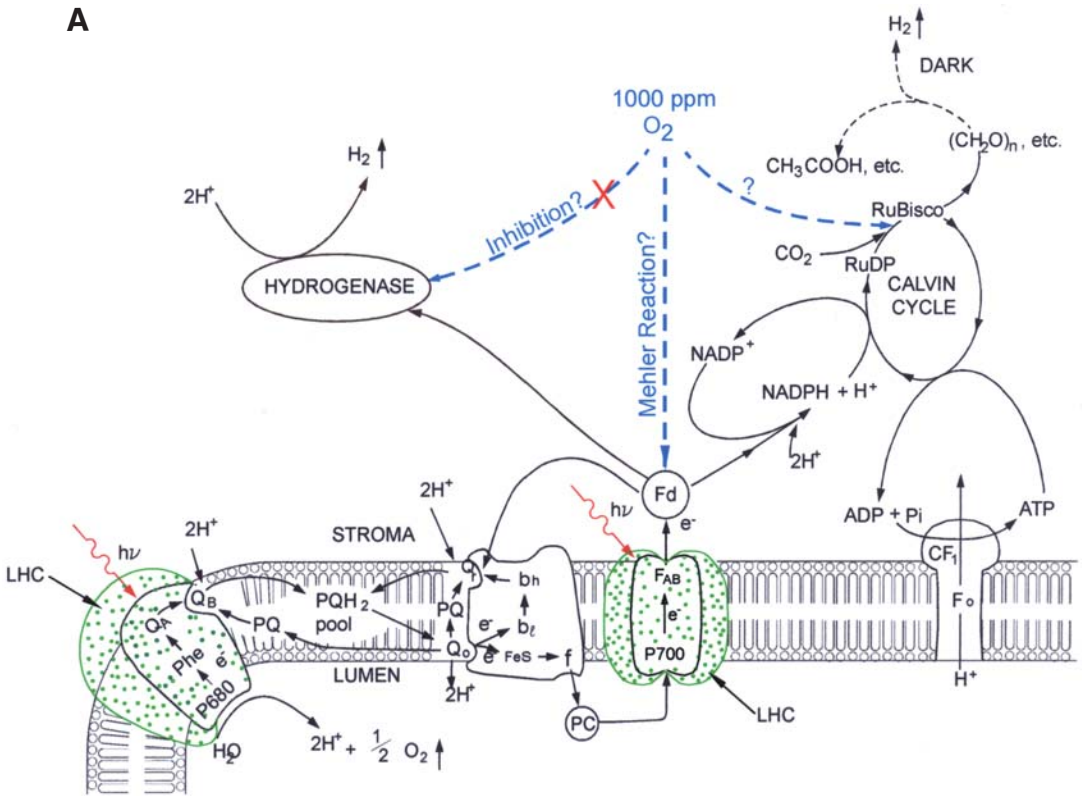

Fig. 5. **(A)** The newly discovered O_2 sensitivity is likely owing to the background O_2 (at about 1000-ppm levels) acting as a terminal sink, in competition with the Fd/hydrogenase H_2 production pathway, for photosynthetically generated electrons. Illustrated by the blue-dotted arrows is a speculated mechanism of how the background O_2 might interact with the photosynthetic H_2 production pathway at the point(s) of RuBisco and/or Fd.

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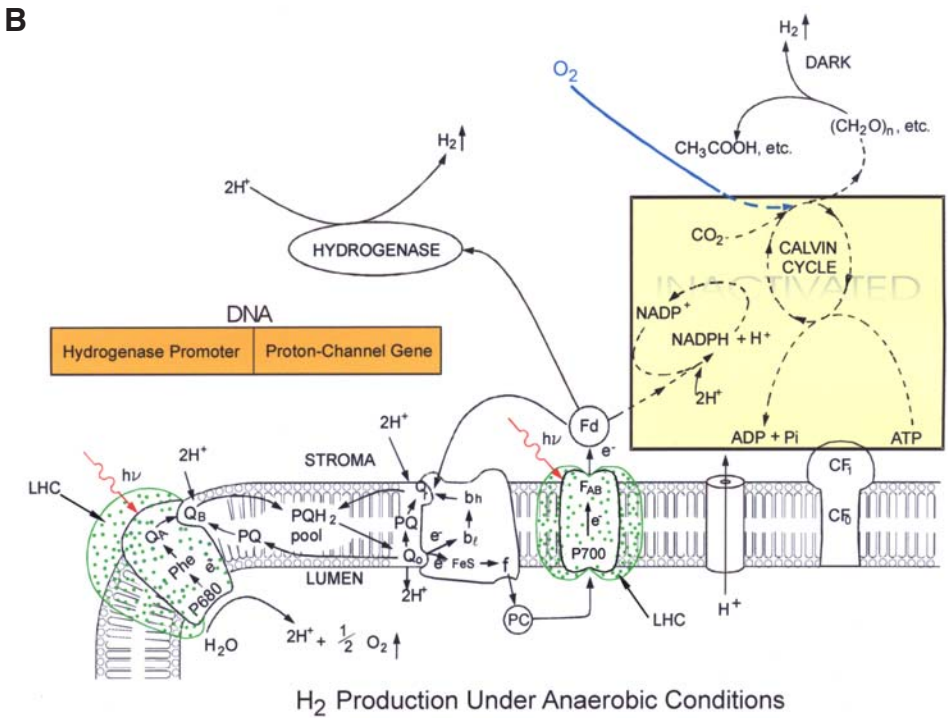

Fig. 5. *(Continued)* **(B)** Development of efficient algal H_2 production system by construction and transformation of vector that contains hydrogenase promoter and a piece of synthetic DNA for the polypeptide proton channel. The transformed alga could grow normally using ambient air CO_2 under aerobic conditions without the polypeptide proton channel, which could be expressed only with the induction of the hydrogenase under anaerobic conditions when its function is needed for enhanced H_2 production. This diagram illustrates a predicted mechanism of how photosynthetic H_2 production could be enhanced through the action of the proposed polypeptide proton channel by dissipation of the static proton gradient across the thylakoid membrane so that the photosynthetic electron transport from PSII water splitting to the Fd/hydrogenase H_2 production pathway can become more efficient, and by inactivation of the Calvin cycle so that both the background O_2 and CO_2 could be prevented from acting as a competitive terminal electron sink.

Potential Application for Enhanced Photosynthetic H_2 Production

The discovery of alternative O_2 sensitivity also provides a new opportunity to develop a more-efficient and robust system for photosynthetic H_2 production. The experimental data with FCCP (Fig. 2) indicate that use of a polypeptide proton channel that does not deactivate the oxidizing equivalents of PSII could enhance H_2 production by eliminating the problems of both the proton-gradient accumulation *(5)* and the newly discovered alternative O_2 sensitivity. Therefore, we propose to create a "designer" photosynthetic organism for production of H_2 by genetic insertion of programmable proton channels in thylakoid membranes. The genetic insertion of programmable thylakoid-membrane proton channels can be achieved by transformation of a host alga with a genetic vector that contains a hydrogenase promoter-linked CF_1 suppressor or membrane polypeptide proton-channel gene. The envisioned "super" alga that can be created in this way should be able to perform autotrophic photosynthesis using ambient air CO_2 as the carbon source and grow normally under aerobic conditions such as in an open pond. When the algal culture is grown and ready for H_2 production, the CF_1 suppressor or proton-channel gene will then be expressed simultaneously with the induction of the hydrogenase enzyme under anaerobic conditions. The expression of the proton-channel gene should produce polypeptide proton channels in the thylakoid membrane, thus dissipating the proton gradient across the thylakoid membrane without ATP formation (Fig. 5B). The expression of the CF_1 suppressor should create CF_0, which may act as a free proton channel without the CF_1 cap, thus similarly dissipating the proton gradient across the thylakoid membrane without ATP formation. The free proton-conductive CF_0 or polypeptide proton channels in the thylakoid membrane could provide two advantages for H_2 photoevolution: (1) accumulation of a proton gradient that impedes photosynthetic electron transport from water to Fd/hydrogenase could be prevented, and (2) the newly discovered O_2-sensitive pathway that competes with the H_2 production pathway for photosynthetically generated electrons could be eliminated.

Therefore, the coexpression of the polypeptide proton channel (or CF_1 suppressor) and hydrogenase genes will make this alga a more-efficient system for production of H_2 by photosynthetic water splitting under anaerobic conditions (Fig. 5B). This organism contains normal mitochondria, which can use reducing power (NADH) from organic reserves (and/or exogenous acetate) to power the cell immediately after returning to aerobic conditions. Therefore, when the algal cell is returned to aerobic conditions after its use under anaerobic conditions for photoevolution of H_2 and O_2, the cell will stop generating free CF_0 (or polypeptide proton channels) in thylakoid membranes and restore its normal photoautotrophic capability by synthesizing functional thylakoids. Consequently, it should be possible to use this type of genetically transformed organism for repeated cycles of

photoautotrophic culture growth under normal aerobic conditions and efficient production of H_2 and O_2 by photosynthetic water splitting under anaerobic conditions.

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